THE EXPERIMENTAL DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER [1]

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HISTORICAL

Although the formation of nitrate from composted nitrogenous organic materials has been long known and practically applied, the current conception that specific biological agencies bring about these natural processes in soils, fresh waters, and the sea was developed only in the last quarter of the nineteenth century. In his studies on acetic acid fermentation Pasteur (1862) anticipated the rôle of bacteria in the ultimate oxidation of ammonia to nitrate, but Schloesing and Müntz (1877 et seq.) first demonstrated the biological nature of the intermediaries. The demonstration was performed in soils; Müntz, shortly afterward (1890), showed that ammonia was produced in soils by microbial breakdown of proteinaceous organic matter. That the oxidation of ammonia to nitrate proceeded through nitrite was distinguished by Munro (1886), but not until Winogradsky (1891, 1892) performed his classic researches were the two oxidations recognized as separately determined by specific ammonia-oxidizing and nitrite-oxidizing bacteria.

Following these researches Adeney (1895) demonstrated the complete sequence of nitrate regeneration in natural and sewage polluted water, establishing the steps by clear-cut quantitative methods. His procedures determined the basis for standard sewage analysis.

It has been held by Brandt (1899, 1902) and many others that the cycle of nitrogen in the sea follows essentially the same phases, and, using this interpretation, a number of researches into the occurrence and distribution of specific marine organisms have been made. These investigations have been reviewed and extended by Waksman, Hotchkiss and Carey (1933). There is considerable conflict in the literature on biological nitrification in the sea, but the conventional picture derived from studies of soils and fresh waters is nevertheless applied to oceanographic chemical data.

Experimental

Because of the growing interest in the regeneration of nitrate in the sea it seemed highly desirable to make a quantitative investigation of the course of organic decomposition and of the simultaneous appearance of nitrogenous products in the water. To this end, a number of experiments were carried out in which a natural source of organic matter—plankton material from customary net-hauls—was allowed to rot and

Table I

Series I.   Source of organic matter: mixed plankton tow.
Microgms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
6–27....	188	0	0	5	5	5	193	150	100
29....	150								160
30....		80	0	5	5	85			204
7– 2....	131	107	0.1	5	5	112	243		180
5....	110	160	3.8	6	2	166	276		123
10....	82						277		
11....		185		10		195			22
15....			2.4						
17....	77						308		40
18....		225	2.7	6	3	231			
24....	40		16				272		
25....		200		32		232			20
27....			55	70	15				
28....		185	80	90	10	275			
30....		125	110	145	35	270			36
8– 1....		60	180	215	35	275			
5....		11	150	275	125	286			
7....	47	0	110	300	190	300	347		
10....		8	2.2	300	298	308			
8–11*....		Portion inoculated with diatoms							
19....	285	0	0	20	20	20	305		
22....	300	0		13	13	13	313		
8–11*....		Control portion, not inoculated							
12....			0.5	360	360				
14....				330					
17....			0.6	330	330				
19....	37	0		360	360	360	397	200	
21....			0.8	330	330				
22....		0							

* One portion inoculated with diatoms and placed in the light.
‡ Does not include dissolved organic nitrogen.

decompose in sea water. Observations were made to follow the disappearance of nitrogen in the decomposing material and its appearance in the water in the form of ammonia, nitrite and nitrate, in the effort to reproduce artificially the cycle of organic decomposition and eventual regeneration. If, by means of the soluble compounds resulting from the decomposition, the water could be rendered fertile and capable of

TABLE II

Series II.   Source of organic matter: mixed plankton tow.
Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
7–12....	400	18	0.2	8	8	26	426	420	82
14....	345								176
15....		>120	0.5	8	8				265
17....	275	>120	0.2	6	6				270
20....	202	550	2.3	8	6	558	760		80
23....		580							
24....			16						43
25....		600		27		627			
27....			60	80	20				
28....		650	110	150	40	800			25
29....	90								
30....		550	240	300	60	850	940		
8– 1....		340	550	590	40	930			16
5....		12	800	820					
7....	78	4	850		60	914	992		
8....				910					
10....		8	550	1000	450	1000			20
12....			190	1100	910	1100			
14....		35	0.1	1050	1050	1100			25
17....			0.1	1100	1100				
18....	133						(1250)		
8–21*....		Portion inoculated with diatoms							
27....	305								
28....			1.0						
31....	840		7.7	320	312	(330)	(1170)		
9– 2....	1010		0	25	25	(35)	(1045)		
3....								470	
8–21*....		Control portion, not inoculated							
22....		7	0.5						
28....			0.3	1050	1050				
29....				1000					
31....			0.5						

* One portion inoculated with diatoms and placed in the light.
‡ Does not include dissolved organic nitrogen.

supporting a new organic growth, comparable to that previously decomposed, the complete cycle would have been carried out. It was also hoped that periodic chemical analysis, by methods now available, might throw light on the sequence of the various steps in the cycle.

The raw material was mixed plankton, collected in the usual way in a No. 20 net, quickly washed, and kept on ice for the few hours before it could be returned to the laboratory. This material was then suspended in 10–15 liters of fresh, filtered sea water and stored in the dark at a temperature of 20–25° C.

Several different analyses were carried out on this material at the start and at intervals thereafter, the various fractions being determined as follows:

1. Total nitrogen contained in the suspended or particulate matter. This is called " plankton nitrogen " in the following descriptions, but also includes bacterial nitrogen as well as that in any other form of suspended matter. This determination follows the procedure described by von Brand (1935) and consists essentially of precipitating the

TABLE III

Series IV. Source of organic matter: mixed plankton tow, strained through No. 8 bolting silk. Micrograms of nitrogen per liter.

Date	Plank-ton	In water					Total in system *	Bacteria in thou-sands per ml.
		Am-monia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *		
7–28	115	43				55	170	152
29			0.6	12	12			200
30	83	110						305
8- 1	63	150	1.0	15	14	165	228	190
5	38	205	<1	10	10	215	253	35
7		190	0.6	10	10	200		16
10	40	200		15	15	215	255	
14		240	0.5	20	20			25
17	41			17	17			
20			13					
21				35				20
22		210	21			(250)		18
27	58	175	120	140	20	315	373	
29			190					
31		29	220	240	20	269		
9- 2		25	225	225	(0)	250		
5	59			260				
8				260				
14		14	310	330	20	344	(400)	
27			130	290	160			
10-4			1					

* Does not include dissolved organic nitrogen.

suspended matter in a sample of the water by the addition of alkali. The precipitate drags down all suspended and colloidal matter, and after settling and centrifugation is separated and resuspended. The resulting small volume of suspension is then used for a determination of total nitrogen by the method of Krogh and Keys (1934): fusion with KOH in a stream of hydrogen, followed by recovery of the ammonia.

2. Ammonia in the water, by a slight modification of the method of Krogh (1934), in which the original design of the still was changed somewhat, to make it more compact and to permit heating electrically.

3. Nitrite in the water by the well-known Griess-Ilosvay method.

4. Nitrate (including nitrite) in the water, by Harvey's reduced

TABLE IV

Series VI.   Source of organic matter: washed, persisting culture of *Nitzchia*. Micrograms of nitrogen per liter.

| Date | Plankton | In water | | | | | Total in system * |
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *	
8–14.............			0.1				
15.............	633	2		17	17	19	652
18.............	514	120	0.1	15	15	165	679
21.............	430	230	0.1	16	16	246	676
27.............	298	310	0.4	24	24	334	632
9– 2.............	201	410	1.3	20	19	430	631
8.............	226			25	25		
14.............		490	0.1	17	17	507	(700)
27.............			0.2	20	20		

* Does not include dissolved organic nitrogen.

strychnine method.   Nitrate then calculated by subtracting the value of nitrite as obtained under 3.

5. Bacterial counts.

The data from the chemical analyses are collected in Tables I to IV and some of them are also presented graphically in Figs. 1 and 2.

Several different " series " were carried out: I, II and IV, in which the organic material was a mixed tow of both phyto- and zoöplankton, of varying amount in each case; and VI, in which a persisting washed culture of diatoms was used as the source of organic matter. In Series II the sea water in which the plankton was suspended came from the surface of Woods Hole Harbor, which probably accounts for its higher content of dissolved organic nitrogen than that of the water in

the other series, which was obtained from the surface a few miles to
the south of Marthas Vineyard.

The rate of decomposition of the plankton varied in the different
series, but after an interval of from 8 to 20 days decomposition stopped.
At this time there was still a constant and not inconsiderable amount of
nitrogen still remaining, either in non-decomposable plankton residues,
bacterial cells, or in other forms. The amount of this residual nitrogen
was from 20 to 35 per cent of that originally determined in the plankton,
or 7 to 10 per cent of that calculated by addition of the different soluble
and insoluble nitrogen fractions.

The rate of decomposition is greatest in the first few days. In
Series II, IV and VI it dropped rather suddenly, but in Series I it fell
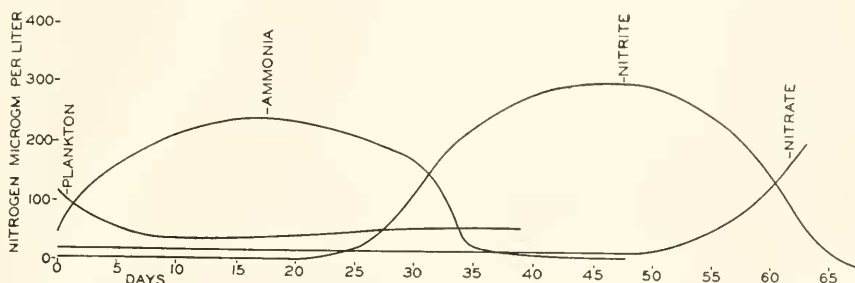gradually over a longer period of time.
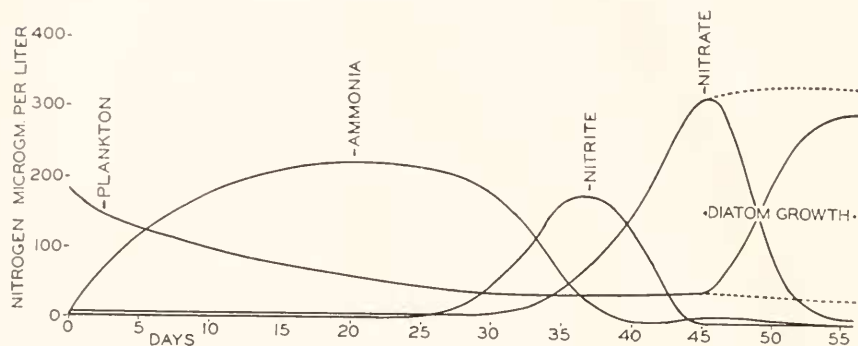
FIG. 1. Series I. The decomposition of nitrogenous organic matter in mixed
plankton, showing the appearance of soluble nitrogen compounds in the water in
which it is suspended. One portion inoculated with diatoms on the forty-fifth day.

Ammonia appeared in the water rapidly and immediately at the be-
ginning of the decomposition. Evidently the liberation of ammonia is
involved in the very first steps in the degeneration of organic matter. It
appears more slowly as time goes on, and reaches a maximum when the
plankton decomposition ends. During this time there is no rise in the
nitrite or nitrate in the water.

The disappearance of ammonia is accompanied by the appearance of
nitrite in the water, and this in turn is eventually oxidized to nitrate.
The phase during which nitrite was present was 15–20 days in Series I
and II, but extended to 40 days in Series IV. In any event, it is not
until nitrite reaches its maximum and starts to disappear that nitrate
increases.

Eventually, however (after 45 days in Series I, 40 days in II, and 65 days in IV), all the available nitrogen was oxidized to nitrate. At this point, in both Series I and II, a portion of the water was placed in the light and inoculated with fresh diatoms, which grew rapidly, raising the " plankton nitrogen " again and lowering the nitrate to its original minimal value.   In this way the complete cycle was carried out.

The question of the existence of soluble nitrogen compounds intermediate between plankton material and ammonia could not be definitely answered, but there was no evidence of any such.   Anything in the nature of colloidal cell-material, partially decomposed, must have been carried down in the precipitation and determined with the rest of the plankton nitrogen.

In the transformation of ammonia to nitrite and nitrate it seems that we are dealing not with an equilibrium of simultaneous processes but with distinct, consecutive steps, each doubtless determined by its own set of conditions.

FIG. 2.   Series IV.   The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended.   Plankton previously filtered through No. 8 bolting silk.

A peculiar situation is revealed by the data from Series I, II and IV. The analyses indicate a larger amount of nitrogen regenerated in soluble forms in the water than that lost by the plankton during decomposition. In the tables the " total nitrogen in the system " has been calculated by addition of all the separate forms determined.   This total shows in general a continuous increase throughout the decomposition, especially in Series II.   That it is not observed in Series VI may be in some way connected with the fact that the organic material in this case consisted of diatoms.

There are three possible explanations for this nitrogen discrepancy :

1. Participation of other forms of nitrogen in the cycle—most plausibly the dissolved organic nitrogen in the water.

2. Systematic errors in either the sampling procedure or the analytical method, so that the values for plankton nitrogen are regularly too low.

3. Nitrogen fixation.

Each of these possibilities has been critically examined and at the present time none of them seems an altogether acceptable explanation. Determinations of the dissolved organic nitrogen in the water, by the method of Krogh and Keys, did not show any significant change during the experiments. Filtering out the larger plankton organisms in Series IV, to lessen the chance of irregular sampling, had no effect. On the other hand, it is true that the decomposition of very much more finely divided organic matter (diatoms) in Series VI yielded only an equivalent amount of ammonia, but this experiment had to be stopped before nitrification began, which is usually the time when the most pronounced difference between added and recovered nitrogen appears.

According to currently accepted views of biological fixation of nitrogen the third possibility seems doubtful.

A further study of this nitrogen discrepancy is now under way.

BACTERIOLOGICAL

The regeneration of inorganic nitrogen from its various organic forms in plankton proceeds through four recognizable stages, each of which is incident to the development and activity of a special bacterial flora. Following the death of plankton organisms, brought about in these experiments by handling, unfavorable temperatures, lack of illumination, crowding, and inadequate nutrient supply, the more labile components of the dead cells undergo hydrolytic cleavage, simultaneously liberating a fraction of the amino nitrogen as ammonia. This breakdown, it would appear from the rate at which ammonia appears and plankton nitrogen diminishes, is not analogous to the digestion of protein by higher animals where individual protein complexes are first split into smaller molecules bearing amino nitrogen.

A part of the decomposition may be autolytic, but it is evident from the tables, which show an attending rapid development of various species of bacteria, that these organisms must play a large part. The absolute numbers are never very high—in fact, not greater than would be expected in freshly taken, unfiltered sea water stored for such intervals of time (Waksman and Renn, 1936; ZoBell and Anderson, 1936). The decline in plankton nitrogen is not so precipitous as the decrease in bacterial numbers. This may mean that the various fractions of plankton nitrogen are not equally susceptible to bacterial attack.

Liberation of ammonia from plankton and other nitrogenous organic matter is, bacteriologically speaking, unspecialized, but is dependent upon the relative proportions of available nitrogen and carbon in the decomposing materials—the materials used in these experiments are, as Redfield (1934) has shown, characteristically nitrogen-rich.

Following the first burst of bacterial activity the ammonia concentration reaches a high level capable of supporting an active nitrite-forming population. Unsuccessful attempts were made to follow the development of the specific bacterial flora responsible for the oxidation of ammonia to nitrite. It appears from the many investigations upon nitrite-forming bacteria in the sea, that the process is due to specific organisms of the *Nitrosomonas* group analogous to the specialized nitrite-forming flora of soils (Waksman, Hotchkiss and Carey, 1933; Carey and Waksman, 1934). The rate at which nitrites are developed here is roughly the same as that observed in cultural studies of the process. Crude cultures prepared by inoculating sea water containing ammonium salts with mud or sea water usually give positive tests for nitrite within ten days or two weeks (depending upon the quantity of inoculum), after which the complete oxidation of ammonia to nitrite proceeds rapidly.

The final stage in nitrogen regeneration progresses more slowly, as is evident from Figs. 1 and 2. Nitrate formation is also effected by a specific bacterial flora, in this case, *Nitrobacter,* an efficient population of which seems to develop at a lower rate than either the non-specific ammonifying or specialized nitrite-forming bacteria. In culture experiments where crude inocula of nitrate-forming bacteria are added to sea water containing nitrite as the only sources of nitrogen, the period of incubation necessary to demonstrate measurable quantities of nitrate ranges from 30 to 90 days. Richer inocula bring about more rapid nitrate formation (Waksman, Hotchkiss and Carey, 1933).

It has been assumed that the relatively late appearance of nitrate in cultures made up of raw sea water to which organic nitrogen in some form has been added is due to the toxic effect of ammonia on nitrate-forming bacteria—a condition that has been clearly demonstrated in experiments on soils. But this relation, if it exists in these experiments, is masked by the slow growth of nitrate-forming bacteria even under favorable conditions. Thus in Series I and II the phase of nitrate formation began promptly after the disappearance of ammonia; in Series IV, on the other hand, it did not begin until 15 days after the ammonia had disappeared.

The regeneration experiments described here certainly are not identical with natural conditions and may not be expected to parallel exactly the courses of the individual phases of nitrate formation in the sea. On

the other hand, they offer a clear picture of their relations.   Further, they validate the conventional conception of the nitrogen cycle as derived from the various studies of the individual processes, by bringing them together in a single summary experiment.

SUMMARY

In conclusion, this study has shown:

1. That it is possible to reproduce the complete cycle of nitrogen regeneration.

2. That the transformation of decomposing plankton (especially diatoms) into ammonia is very rapid, beginning as soon as the initial substance disappears from the body.   The amounts of soluble nitrogenous substances of higher molecular weight in these experiments can have been only very small.

3. The main stages in the decomposition are: dead body—ammonia —nitrite—nitrate.

4. Under these conditions, at least, no toxic substances are formed which inhibit the flowering of diatoms.   The rapid and abundant development of the latter showed the regeneration of the water to be complete.

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